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Diadenosine polyphosphates regulate cytosolic calcium in human fibroblast cells by interaction with P_{2x} purinoceptors coupled to phospholipase C

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Abstract

The effects of diadenosine pentaphosphate (AP_5A), and diadenosine hexaphosphate (AP_6A) on the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) were evaluated in cultured human fibroblast cells (HF cells) using the fluorescent dye technique. AP_5A, and AP_6A concentration-dependently increased $[Ca^{2+}]_i$ in HF cells. The addition of 10 μmol/l AP_5A and AP_6A significantly increased $[Ca^{2+}]_i$ in HF cells from 71 ± 3 nmol/l ($n = 184$) to 241 ± 39 nmol/l ($n = 11$; $P < 0.001$ compared to resting value) and to 227 ± 26 nmol/l ($n = 23$; $P < 0.001$), respectively. The purinoceptor P_2 blockers, suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), inhibited the diadenosine polyphophate-induced $[Ca^{2+}]_i$ increase, whereas the P_{2y} purinoceptor blocker, reactive blue, had no effect. Adenosinetriphosphate (ATP) and the P_{2x} agonist, α,β-methylene-ATP also significantly increased $[Ca^{2+}]_i$ in HF cells, whereas the P2y agonist methylthio-ATP showed only a small $[Ca^{2+}]_i$ response. Diadenosine polyphosphates mainly induced transplasmamembrane Ca^{2+} influx as was confirmed by experiments in the absence of extracellular Ca^{2+} or by manganese quenching studies. Organic (verapamil) and inorganic Ca^{2+} channel blockers ($NiCl_2$) significantly reduced the AP6A induced transplasmamembrane Ca^{2+} influx. The inhibitor of phosphatidylcholine-specific phospholipase C, D609, significantly reduced the effect of diadenosine polyphosphates on $[Ca^{2+}]_i$ in HF cells. It is concluded that diadenosine polyphosphates regulate transplasmamembrane Ca^{2+} influx after occupation of P_{2x} receptors via activation of phosphatidylcholine-specific phospholipase C and hence of voltage-operated Ca^{2+} channels.

Keywords: Diadenosine polyphosphate; Calcium ion, cytosolic free; Fibroblast; Phospholipase C; (Human)

1. Introduction

Biological responses to extracellular adenine nucleotides include increases in membrane permeability, stimulation of exocrine and endocrine secretion, relaxation or contraction of smooth muscles, platelet aggregation, and modulation of neural excitability [1,2]. Recently novel vasoconstrictor agents, diadenosine pentaphosphate (AP_5A) and diadenosine hexaphosphate (AP_6A) have been isolated from human platelets [3]. Both AP_5A and AP_6A increased the systemic blood pressure in rats [3]. AP_5A and AP_6A have also been shown to stimulate growth in glomerular mesangial cells [4] and to be released by platelet aggregation [5]. Therefore, both agents may contribute to the development of glomerulosclerosis.

In this context, the effects of AP_5A and AP_6A on fibroblasts are also of interest, but have not yet been studied in detail. In the present study, it was examined whether and how human fibroblasts are stimulated by AP_5A and AP_6A. Moreover, our knowledge on the responsiveness of human tissue to diadenosine polyphosphates still needs to be amplified, since up to now only human umbilical cord arteries are known to be contracted by AP_5A and AP_6A [6]. The results of the present study indicate that human fibroblasts are responsive to AP_5A and AP_6A and that stimulation of fibroblasts by these diadeno-

Abbreviations: AP_5A, diadenosine pentaphosphate; AP_6A, diadenosine hexaphosphate; $[Ca^{2+}]_i$, cytosolic-free Ca^{2+} concentration; HF cells, human fibroblast cells; D609, phosphatidylcholine-specific phospholipase C inhibitor; DPCPX, dipropylcyclopentylxanthine; DMPX, dimethylpropylxanthine; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; α,β-methylene-ATP, α,β-methylene-adenosine triphosphate; methylthio-ATP, methylthio-adenosine triphosphate

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sine polyphosphates is mediated partly by P_{2x} purinoceptors and partly by suramin-insensitive diadenosine polyphosphate receptors.

2. Materials and methods

2.1. Cell culture

Human fibroblasts (HF cells) were obtained from healthy subjects and cultured by the tissue explant method. Briefly, cells were incubated in Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany), containing 10% (v/v) fetal calf serum (Boehringer, Mannheim, Germany), 100 U/ml penicillin G and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed initially after 24 h and then every 2–3 days. After the first subculture, cells were subcultured every week at a seeding density of about $1 \cdot 10^4$ cells/cm^2 and reached confluence in 8 to 10 days. Thereafter they were harvested by adding 0.05% trypsin, and the culture was continued up to 8 passages.

It was confirmed that cultured HF cells were free from contamination with endothelial cells. A viability of HF cells higher than 95% was observed by trypan blue exclusion. Cells were made quiescent by incubation in serum-free medium containing 0.1% bovine serum albumin, 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h prior to measurements of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$).

2.2. Measurement of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in HF cells

Measurements of $[Ca^{2+}]_i$ were performed using the Ca^{2+}-sensitive dye 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′N′*-tetra-acetic acid, pentaacetoxymethylester (fura2/AM) as described by Grynkiewicz et al. [7] and Williams et al. [8] using HF cells grown on round coverslips with a diameter of 13 mm. HF cells on cover slips ($2.5 \cdot 10^4$ cells/cover slip) were washed twice in physiological salt solution (PSS, containing in mmol/l: NaCl 135, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 5.5, *N*-2-hydroxyethyl-piperazine-*N′*-2-ethanesulfonic acid (Hepes) 10, pH 7.4) and then incubated with 10 μmol/l cell-permeant fura2/AM and 0.02% (w/v) nonionic surfactant polyoxypropylenepolyoxyethylene block copolymer, pluronic F-127 (Molecular Probes, Eugene, OR), for 60 minutes at 37°C. After loading of the cells with fura2/AM the experiments were continued only when a viability higher than 95% was observed by trypan blue exclusion. At the end of the loading period, the coverslips were washed twice in PSS and inserted into quartz glass cuvettes with 2 ml PSS. The fluorescence intensity of fura2-loaded HF cells was measured at 37°C using a spectrofluorophotometer RF-5001 PC (Shimadzu, Tokyo, Japan) The fluorescence of fura2 was measured using a data sampling interval of 1 s with alternate excitation wavelengths of 340 nm and 380 nm (bandwidth, 5 nm), and emission was collected at 510 nm (bandwidth, 5 nm). Autofluorescence was measured in similar cells which had not been loaded with fura2-acetoxymethylester and was less than 5% of the total fluorescence of fura2 loaded HF cells. After the subtraction of autofluorescence for each wavelength, the ratio (R) of the measured fluorescence values at 340 nm and 380 nm excitation was calculated [7,8]. The $F_{340\ nm}/F_{380\ nm}$ excitation ratio of resting HF cells remained constant during the whole experiment, indicating a stable resting $[Ca^{2+}]_i$ in HF cells. As indicated by control experiments no significant spontaneous increase of resting $[Ca^{2+}]_i$ could be observed during the measurements. Calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was performed with digitonin and ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid (EGTA) at each cover slip. 1 mmol/l digitonin and 5 mmol/l EGTA were sequentially added to determine the maximum (R_{max}) and the minimum (R_{min}) of the $F_{340\ nm}/F_{380\ nm}$ excitation ratio, respectively. Control experiments confirmed that further increase of the digitonin or EGTA concentration had no effect on R_{max} or R_{min}, respectively. The minimal fluorescence excitation ratio was 0.99 ± 0.01 and the maximal fluorescence excitation ratio was 2.77 ± 0.11. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. with a dissociation constant of fura2 for Ca^{2+} of 224 nmol/l [7]. Leakage during the measurement was less than 5% of the total fluorescence as observed by quenching of external fluorescence with 100 μmol/l $MnCl_2$ as described elsewhere [9].

Since the purinoceptor P2 blocker suramin may interfere with the fura2 fluorescence additional experiments were carried out using the calcium-sensitive fluorescent dye fluo3 (Sigma) using an excitation wavelength of 490 nm and an emission wavelength of 525 nm when suramin was used.

2.3. Ca^{2+} influx measurements using $MnCl_2$

To measure Mn^{2+} influx as a reporter of unidirectional Ca^{2+} influx, the entry of Mn^{2+} into HF cells was evaluated using the fura2 quenching technique [10]. HF cells were loaded as for $[Ca^{2+}]_i$ measurements and fura2 fluorescence monitored in a Ca^{2+} containing medium at the Ca^{2+} insensitive excitation wavelength 360 nm. Then, 0.5 mmol/l $MnCl_2$ was added, and its entry into HF cells was measured as the rate of fluorescence decrease.

2.4. Purification of the diadenosine phosphates AP_5A and AP_6A

As, both AP_5A and AP_6A showed impurities in commercially available preparations, both agents were only used after purification with HPLC [4].

Diadenosine pentaphosphate (AP_5A) and diadenosine hexaphosphate (AP_6A), adenosine triphosphate (ATP), α,β-methylene-ATP, verapamil, the phosphatidylcholine-specific phospholipase C inhibitor, tricyclodecan-9-yl xanthogenate (D609), and all other substances were purchased from Sigma Chemical Co. (Deisenhofen, Germany) if not indicated otherwise. The purinoceptor P2 blockers, suramin, and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), the purinoceptor P2y blocker, reactive blue, the P2y receptor agonist, methylthio-ATP, the adenosine A1 receptor blocker, dipropylcyclopentylxanthine (DPCPX), and the adenosine A2 receptor antagonist, dimethylpropylxanthine (DMPX), were purchased from Research Biochemical International (Cologne, Germany).

2.5. Statistics

All data are presented as mean ± SE. For statistical evaluation of the data Wilcoxon's test was used. Two-tailed P values less than 0.05 were considered to be significant. Original tracings shown in the figures are representative for at least 5 separate experiments.

3. Results

3.1. Effect of diadenosine polyphosphates on $[Ca^{2+}]_i$ in HF cells

Resting $[Ca^{2+}]_i$ in HF cells was 71 ± 3 nmol/l (n = 184). The administration of diadenosine polyphosphates increased $[Ca^{2+}]_i$ in HF cells. As shown in Fig. 1, the addition of AP_5A and AP_6A instantly increased $[Ca^{2+}]_i$, reaching a peak concentration after about 30 to 50 s. Thereafter the $[Ca^{2+}]_i$ declined slowly, reaching a sustained $[Ca^{2+}]_i$ level above the resting value. As shown in Fig. 2, the administration of diadenosine polyphosphates increased $[Ca^{2+}]_i$ in HF cells in a concentration-dependent manner. From these curves the apparent EC 50 values

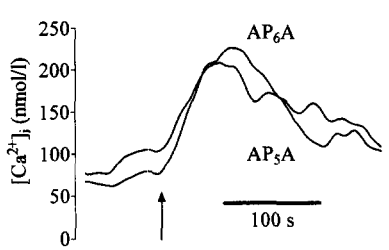

Fig. 1. Effect of diadenosine pentaphosphate (AP_5A) and diadenosine hexaphosphate (AP_6A) on cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human fibroblast cells (HF cells). HF cells were loaded with the fluorescent dye fura2 and $[Ca^{2+}]_i$ was determined spectrophotometrically. At the indicated time 10 μmol/l AP_5A or AP_6A were added, respectively. The figure shows representative tracings out of 11–23 similar experiments.

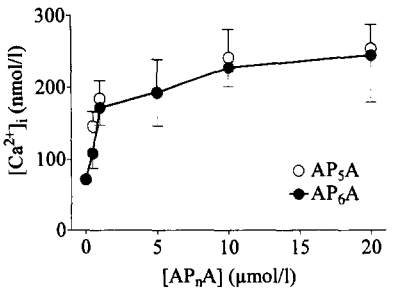

Fig. 2. Concentration-reponse curve of diadenosine pentaphosphate (AP_5A; open circles) and diadenosine hexaphosphate (AP_6A; filled circles)-induced increase of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human fibroblast cells (HF cells). AP_5A or AP_6A were added to fura2-loaded HF cells and the peak $[Ca^{2+}]_i$ increase was determined. Values represent the mean ± SEM.

were 0.58 μmol/l and 0.29 μmol/l for AP5A and AP6A, respectively. The addition of 10 μmol/l AP_5A and AP_6A significantly increased $[Ca^{2+}]_i$ in HF cells to 241 ± 39 nmol/l (n = 11; $P < 0.001$ compared to resting value) and to 227 ± 26 nmol/l (n = 23; $P < 0.001$), respectively. HF were subcultured up to the 8th passage. The AP_6A-induced $[Ca^{2+}]_i$ increase was not significantly different between different subcultures. The $[Ca^{2+}]_i$ increase induced by 10 μmol/l AP_6A was 221 ± 46 nmol/l in HF cells from passage 2 and it was 196 ± 32 nmol/l in passage 8 (n.s.). The sustained $[Ca^{2+}]_i$ after the administration of AP_5A and AP_6A were 182 ± 44 nmol/l (n = 11; $P <$ 0.001) and 170 ± 18 nmol/l (n = 23, $P < 0.001$).

3.2. Diadenosine polyphosphates induce Ca^{2+} influx

In order to evaluate whether the diadenosine polyphosphate-induced $[Ca^{2+}]_i$ increase is due the transplasmamembrane Ca^{2+} influx, experiments were conducted in the absence of external Ca^{2+} or using the manganese quenching technique. In the absence of external Ca^{2+} the administration of 10 μmol/l AP_5A and AP_6A did not significantly change $[Ca^{2+}]_i$ in HF cells (57 ± 11 nmol/l, n = 5; and 37 ± 4 nmol/l, n = 4), indicating that the diadenosine polyphosphate-induced $[Ca^{2+}]_i$ increase in the presence of external Ca^{2+} was due to a transplasmamembrane Ca^{2+} influx. In additional experiments Mn^{2+} influx was measured as a reporter of unidirectional Ca^{2+} influx. As shown in Fig. 3, the administration of 10 μmol/l AP_6A produced a rapid decrease of fura2 fluorescence due to transplasmamembrane Mn^{2+} influx. Similar results could be obtained with AP_5A. These findings support the view that diadenosine polyphosphates enhance the Ca^{2+} entry into HF cells.

3.3. Effect of purinoceptor blockers on diadenosine polyphosphate-induced $[Ca^{2+}]_i$ increase

Next the question arises whether the $[Ca^{2+}]_i$ increase in HF cells induced by diadenosine polyphosphates is medi-

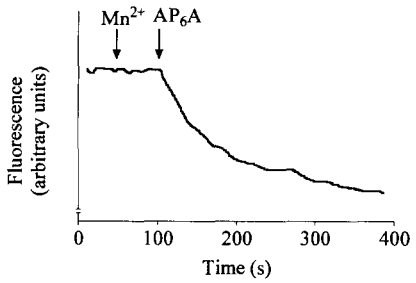

Fig. 3. Original tracing showing the diadenosine hexaphosphate (AP_6A)-induced Mn^{2+} entry into cultured human fibroblast cells (HF cells) as measured by fura2 quenching. 10 μmol/l AP_5A were added to fura2 loaded HF cells in the presence of Mn^{2+} and the fluorescence was measured at the Ca^{2+} insensitive excitation wavelength of 360 nm. The tracing indicating the reduction of intracellular fura2 fluorescence due to transplasmamembrane Mn^{2+} influx is representative for 5 similar experiments.

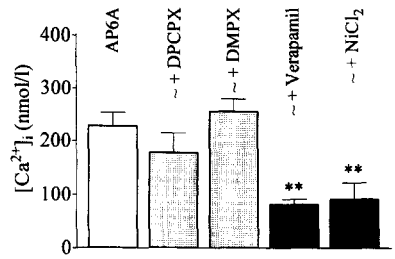

Fig. 5. Effect of adenosine receptor antagonists or calcium channel blockers on diadenosine hexaphosphate (AP6A) induced increase of cytosolic-free calcium concentration ($[Ca^{2+}]_i$) in cultured human fibroblast cells (HF cells). The adenosine A1 receptor antagonist, dipropylcyclopentylxanthine (DPCPX), the adenosine A2 receptor antagonist, dimethylpropylxanthine (DMPX), the organic calcium channel blocker, verapamil, or the inorganic calcium channel blocker, NiCl2, were added prior to the administration of 10 μmol/l AP6A. Data represent the mean ± SEM of the $[Ca^{2+}]_i$ increase after administation of AP6A from 5 to 23 experiments. ** $P < 0.01$ compared to control conditions (open bar).

ated by purinergic receptors. The AP_6A-induced $[Ca^{2+}]_i$ increase was decreased after administration of the purinoceptor P_2 blockers, suramin (Fig. 4) or PPADS, indicating that the effects of AP_6A may partly be transmitted by purinergic P_2 receptors. Suramin reduced the AP_6A-induced peak $[Ca^{2+}]_i$ from 227 ± 26 nmol/l to 102 ± 20 nmol/l ($n = 5$; $P < 0.01$). To avoid any interference of suramin with the fluorescent dye we loaded HF cells with the calcium-sensitive fluorescent dye fluo3 when suramin was used. Under these conditions it was confirmed that the purinoceptor P_2 blocker suramin reduced the AP6A induced $[Ca^{2+}]_i$ increase in HF cells. PPADS reduced the AP_6A-induced peak $[Ca^{2+}]_i$ from 227 ± 26 nmol/l to 155 ± 33 nmol/l ($n = 5$). On the other hand, the inhibition of purinergic P_{2y} receptors by reactive blue did not significantly reduce the AP_6A-induced peak $[Ca^{2+}]_i$ (284 ± 50 nmol/l, $n = 5$, $P = 0.238$).

3.4. Effect of adenosine receptor blockers on diadenosine polyphosphate-induced $[Ca^{2+}]_i$ increase

The adenosine A_1 receptor blocker, DPCPX, did not significantly reduce the $[Ca^{2+}]_i$ increase induced by AP_5A or AP_6A. Similarly, the adenosine A_2 receptor antagonist, DMPX, did not significantly reduce the AP_6A-induced $[Ca^{2+}]_i$ increase in HF cells (Fig. 5). DPCPX or DMPX had no effect on the sustained $[Ca^{2+}]_i$ increase after the addition AP_5A or AP_6A.

3.5. Effect of calcium antagonists on diadenosine polyphosphates induced $[Ca^{2+}]_i$ increase

The Ca^{2+} channel blockers verapamil (final concentration: 10 μmol/l) or $NiCl_2$ (final concentration: 6 mmol/l) significantly reduced the peak $[Ca^{2+}]_i$ after AP_6A administration (Fig. 5). Verapamil and $NiCl_2$ significantly reduced the sustained $[Ca^{2+}]_i$ in HF cells after addition of AP_6A from 170 ± 18 nmol/l ($n = 23$) to 77 ± 11 nmol/l ($n = 5$, $P < 0.01$) or to 53 ± 11 nmol/l ($n = 5$, $P < 0.01$), respectively. These experiments indicate that voltage operated Ca^{2+} channels may be involved in the diadenosine polyphosphate induced transplasmamembrane Ca^{2+} influx.

3.6. Inhibition of the phosphatidylcholine-specific phospholipase C reduces the diadenosine polyphosphates induced $[Ca^{2+}]_i$ increase

After administration of the phosphatidylcholine-specific phospholipase C inhibitor, D609, the AP_6A induced peak

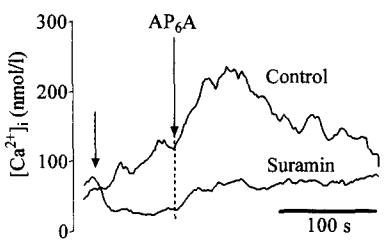

Fig. 4. Original tracing showing the effect of suramin (100 μmol/l) on diadenosine hexaphosphate (AP_6A)-induced increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human fibroblast cells (HF cells). Original tracings are representative of 5 similar experiments.

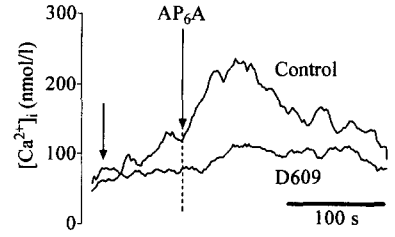

Fig. 6. Effect of the phosphatidylcholine-specific phospholipase C inhibitor, D609, on diadenosine hexaphosphate (AP_6A)-induced increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human fibroblast cells (HF cells). Original tracings are representative of 5 similar experiments.

$[Ca^{2+}]_i$ increase was significantly reduced from 227 ± 26 nmol/l to 112 ± 27 nmol/l ($n = 5$, $P < 0.01$) and the sustained $[Ca^{2+}]_i$ increase was reduced from 170 ± 18 nmol/l ($n = 23$) to 62 ± 21 nmol/l (Fig. 6, $n = 5$, $P < 0.01$). These experiments indicate that the transplasmamembrane Ca^{2+} influx subsequent to the administration of diadenosine polyphosphates is mediated by phospholipase C.

In addition, the effects of ATP and α,β-methylene-ATP on $[Ca^{2+}]_i$ in HF cells were studied. 10 μmol/l ATP significantly increased $[Ca^{2+}]_i$ in HF cells to 451 ± 91 nmol/l ($n = 9$, $P < 0.001$ compared to control value), and the sustained $[Ca^{2+}]_i$ was 260 ± 57 nmol/l. ($n = 9$, $P < 0.01$). In the presence of suramin the ATP-induced peak $[Ca^{2+}]_i$ was significantly reduced to 143 ± 26 nmol/l ($n = 5$, $P < 0.02$) and the sustained $[Ca^{2+}]_i$ was reduced to 124 ± 28 nmol/l (Fig. 5, $n = 5$). The purinoceptor P_{2x} agonist, α,β-methylene-ATP, significantly increased $[Ca^{2+}]_i$ in HF cells to 163 ± 30 nmol/l ($n = 5$, $P < 0.01$), and the sustained $[Ca^{2+}]_i$ was 160 ± 33 nmol/l ($n = 5$, $P < 0.01$). The purinoceptor P_{2y} agonist, methylthio-ATP, showed a small $[Ca^{2+}]_i$ increase (peak $[Ca^{2+}]_i$ 110 ± 14 nmol/l, $n = 5$, $P < 0.05$), whereas the sustained $[Ca^{2+}]_i$ was 81 ± 10 nmol/l ($n = 5$).

To further examine the interactions between ATP and AP_5A or AP_6A on $[Ca^{2+}]_i$, either AP_5A or AP_6A were administered after preincubation with 10 μmol/l ATP. Under these conditions both diadenosine polyphosphates induced a further $[Ca^{2+}]_i$ increase (AP_5A: $[Ca^{2+}]_i$ increase 63 ± 11 nmol/l, $n = 5$; AP_6A: $[Ca^{2+}]_i$ increase 57 ± 15 nmol/l, $n = 5$).

4. Discussion

The present findings demonstrate that diadenosine polyphosphates induce a Ca^{2+} influx in HF cells. The effects of diadenosine polyphosphates on HF cells were studied firstly, to evaluate whether also fibroblasts, besides mesangial cells [4] and vascular smooth muscle cells [3], respond to diadenosine polyphosphates, and secondly, to test the responsiveness of a further human cell type to diadenosine polyphosphates.

The experiments show that the increase in $[Ca^{2+}]_i$ induced by diadenosine polyphosphates is almost exclusively due to a transplasmamembrane Ca^{2+} influx, whereas Ca^{2+} release from cellular stores does not appear to be involved in diadenosine polyphosphate-induced changes of $[Ca^{2+}]_i$ in HF cells. In other cell types, diadenosine polyphosphates also elicit a release of intracellular stored Ca^{2+} [11,12].

The experiments with organic and inorganic Ca^{2+} channel blockers indicate that the diadenosine polyphosphate-induced Ca^{2+} influx may not exclusively be mediated by voltage-dependent L-type Ca^{2+} channels. Interestingly, a considerable portion of the diadenosine polyphosphate-induced Ca^{2+} influx was insensitive to organic and inorganic Ca^{2+} channel blockers. This finding is consistent with the concept that P_{2x} receptors represent a non-selective cation channel activated by nucleotides, which is distinct from the L-type Ca^{2+} channel [13].

The response of HF cells to AP_5A and AP_6A is partly, but not entirely mediated by P_{2x} purinoceptors. In the experiments with the unspecific P_2 purinoceptor blocker, suramin, and with the specific P_{2x} purinoceptor blocker, PPADS, a significant portion of the $[Ca^{2+}]_i$ response was insensitive to purinoceptor blockade. Pintor and Miras Portugal recently demonstrated a novel diadenosine polyphosphate receptor in rat brain synaptosomes, which, unlike to the known P_2 purinoceptors, is insensitive to suramin [14]. Therefore it may be speculated that also in human fibroblasts this diadenosine polyphosphate receptor may be activated. Although hitherto this receptor cannot be characterized using a specific blocker, the activation of such a receptor is the most likely explanation of the partial insensitivity of the diadenosine polyphosphate response to P_2 purinoceptor blockers.

Besides specific diadenosine polyphosphate receptors, also P_{2x} purinoceptors mediate the diadenosine polyphosphate response in HF cells, as evidenced by the inhibitory effects of suramin and PPADS. Furthermore, ATP and α,β-methylene ATP, but not the P_{2y} purinoceptor agonist elicited a similar response. Furthermore, the diadenosine polyphosphates tested and ATP at least partially activate different receptors, since the administration of diadenosine polyphosphates after preincubation with a maximally effective concentration of ATP yielded a further $[Ca^{2+}]_i$ increase. This finding also makes the hypothesis unlikely, that diadenosine polyphosphates exert their action through their degradation products such as ATP.

The experiments further allow some speculations on the signaling events downstream to the receptor. Firstly the findings suggest that the phosphatidylcholine-specific phospholipase C be involved. Since this enzyme is known to produce diacylglycerol and phosphorylcholine, which are known to modulate Ca^{2+} influx, this finding fits well with the results showing the predominant role of Ca^{2+} influx for the $[Ca^{2+}]_i$ response.

In summary, the study demonstrates that AP_5A and AP_6A stimulate human fibroblasts as shown by the $[Ca^{2+}]_i$ response. The $[Ca^{2+}]_i$ increase is partly P_{2x} purinoceptor-mediated and may partly be due to activation of a specific diadenosine polyphosphate receptor. As both agents are released by platelet aggregation [5], possibly AP_5A and AP_6A play a role in local stimulation of fibroblasts and hence may modulate fibrotic processes.

Acknowledgements

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References

[1] Boeynaems, J.-M. and Pearson, J.D. (1990) Trends Pharmacol. Sci. 11, 34–37.
[2] Burnstock, G. (1991) Nucleosid. Nucleotid. 10, 917–930.
[3] Schlüter, H., Offers, E., Brüggemann, G., van der Giet, M., Tepel, M., Nordhoff, E., Karas, M., Spieker, C., Witzel, H. and Zidek, W. (1994) Nature 367, 186–188.
[4] Heidenreich, S., Tepel, M., Schlüter, H., Harrach, B. and Zidek, W. (1995) J. Clin. Invest. 95, 2862–2867.
[5] Agha, A., Schlüter, H., König, S., Biel, K., Tepel, M. and Zidek, W. (1992) J. Vasc. Res. 29, 281–289.
[6] Davies, G., MacAllister, R.J., Bogle, R.G. and Vallance, P. (1995) Br. J. Clin. Pharmacol. 40, 170–172.
[7] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[8] Williams, D.A., Fogarty, K.E., Tsien, R.Y. and Fay, F.S. (1985) Nature 318, 558–561.
[9] Simonson, M.S. and Dunn, M.J. (1991) Exp. Cell Res. 192, 148–156.
[10] Demaurex, N., Lew, D.P. and Krause, K.H. (1992) J. Biol. Chem. 267, 2318–2324.
[11] Castro, E., Pintor, J. and Miras-Portugal, M.T. (1992) Br. J. Pharmacol. 106, 833–837.
[12] Green, A.K., Dixon, C.J., McLennan, A.G., Cobbold, P.H. and Fisher, M.J. (1993) FEBS. Lett. 322, 197–200.
[13] Dalziel, H.H. and Westfall, D.P. (1994) Pharmacol. Rev. 46, 449–466.
[14] Pintor, J. and Miras-Portugal, M.T. (1995) Br. J. Pharmacol. 115, 895–902.